Investigating the Effect of Mixing Ratio on Molar Mass Distributions of Synthetic Polymers determined by MALDI-TOF Mass Spectrometry using Design of Experiments

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It is well known that the mixing ratio affects the molar mass distribution of synthetic polymers determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Surely, the molar mixing ratio determines whether a mass spectrum will be obtained or not. However, depending on the mass range, several effects such as multimer formation occur, which might be a source of errors in molar mass distribution calculations. In this study, the effect of mixing ratio was investigated for several synthetic polymers, including polystyrene (PS), poly(dimethylsiloxane) (PDMS), poly(ethylene glycol) (PEG), and poly(methyl methacrylate) (PMMA) using statistical designs of experiments. The 2^3 full factorial design was found to be suitable in the study of more than 1000 samples. The obtained MALDI mass spectra as well as the ANOVA statistics show that the mixing ratio affects the molar mass distribution. The optimal mixing ratio for a defined synthetic polymer depends on the studied combination (matrix, cationization reagent, solvent). (J Am Soc Mass Spectrom 2010, 21, 1870–1875) © 2010 American Society for Mass Spectrometry

MALDI-TOF mass spectrometry has become a powerful analytical tool for the study of synthetic polymers though its mechanism is still not well understood. In recent years many factors influencing the quality of MALDI mass spectra of synthetic polymers were investigated especially factors of sample preparation. It has been shown that the matrix type, cationization reagent, their concentrations, as well as the type of solvent or solvent mixture, and the spotting technique affect the polymer distribution [1–16]. Besides sample preparation, the instrumentation parameters influence the determined molar mass distribution [17, 18]. To find the optimal instrumental parameters Wallace et al. [19] applied a specialized noise-adapted filtering method. Wetzel et al. [20] studied significant instrument parameters for optimization of MALDI-TOF analysis employing a 2^{5-1} fractional factorial design while Liland et al. [21] had used a 2^3 full factorial design and fractional factorial designs. By contrast, optimization of sample preparation had been based on trial and error until now.

The proper choice of matrix, cationization reagent, and solvent plays the key role in sample preparation. Matrix and solvent can be selected according to the chemical nature of the polymer [10, 12, 22–24]; for the selection of cationization reagents the application of HSAB (hard/soft acid/base) principle was suggested

[25, 26]. Several investigations of the effect of mixing ratio had been carried out and comprehensively discussed [2, 27, 28]. However, the most investigations are limited to the analyte/matrix ratio. Only Hoteling [29] mentioned the analyte/salt ratio and matrix/salt ratio, too. But, to our knowledge, there is no publication which dealt with a systematic research investigating the polymer/matrix/cationization reagent ratio.

In this study, for different synthetic polymers we had investigated the effect of mixing ratio in MALDI-TOF MS analyzing various combinations (matrix, cationization reagent, and solvent) employing the dried-droplet preparation method with the single solvent approach [3]. We have applied design of experiments to optimize the mixing ratio of each combination of matrix, cationization reagent, and solvent. Initially, we investigated the suitability of different experimental designs, including a Box-Behnken design, a 2^3 full factorial design, and a 2^{3-1} fractional factorial design. Subsequently, we applied the most suitable experimental design, the 2^3 full factorial design, for MALDI analysis of synthetic polymers in the mass range up to 30,000 Da.

Experimental

Samples and Reagents

Polystyrenes PS2850 (PDI = 1.04), PS5460 (PDI = 1.03), PS11600 (PDI = 1.03), and PS21000 (PDI = 1.02) as well as PMMA7800 (PDI = 1.14) were obtained from

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Macherey-Nagel (Düren, Germany). PDMS1110 (PDI = 1.37) was obtained from PSS (Mainz, Germany). PEG4820 (PDI = 1.04), PEG9230 (PDI = 1.08), and PS30250 (PDI = 1.01) were obtained from Varian Inc. (Church Stretton, UK). Higher molar mass PDMS were manufactured by Wacker Chemie AG and fractionated by SEC in our laboratory. For this study, frequently used MALDI matrices were 2-[(2E)-3-(4-*tert*-butylphenyl)-2-methylpropenylidene]malononitrile (DCTB), 2',6'-dihydroxyacetophenone (DHAP), 2,5-dihydroxybenzoic acid (DHB), 2,5-dihydroxy-1,4-benzoquinone (DHBQ), *trans,trans*-1,4-diphenyl-1,3-butadiene (DPBD), dithranol (DT), nicotinic acid (NA), and 2',4',6'-trihydroxyacetophenone (THAP). Silver trifluoroacetate, lithium trifluoroacetate, copper (II) trifluoroacetate, lithium chloride, and sodium nitrate were used as cationization reagents. MALDI matrices and cationization reagents were purchased from Sigma-Aldrich (Steinheim, Germany) and used as received. Toluene, dimethyl carbonate, acetone, ethanol, pyridine, acetonitrile, chloroform, dichloromethane, 1,4-dioxane, methyl ethyl ketone, methyl isobutyl ketone, *N,N*-dimethylformamide, and tetrahydrofuran, stabilized with 2,6-di-*tert*-butyl-4-methylphenol, purchased from Merck (Darmstadt, Germany), were used as solvents in the experiments.

MALDI-TOF MS

All experiments were carried out on an AXIMA Performance MALDI-TOF mass spectrometer (Shimadzu Biotech, Manchester, UK) equipped with a nitrogen laser ($\lambda = 337$ nm). The mass spectra were obtained in linear mode with an accelerating voltage of 20 kV; at least 150 to 200 mass spectra were averaged and baseline correction was performed. Although Wetzel et al. [20] showed that detector voltage can have a high impact on S/N ratios, none of the voltage settings were manually changed. As shown in numerous papers, laser energy has a high impact on MALDI-TOF MS analysis. Thus, in this study, laser energy was optimized for each sample. The laser energy was set up for each measurement slightly above threshold of the analyte signal.

Sample Preparation

More than 1000 samples (different synthetic polymers were analyzed using various combinations of matrix, cationization reagent and solvent) were prepared according to the single solvent approach, i.e., matrix, cationization reagent, and polymer were dissolved in the same solvent. Polymer solutions were prepared at a concentration of 5 mg/mL, matrix solutions at a concentration of 10 mg/mL, cationization reagent solutions at a concentration of 0.1 mol/L. According to Table 1, the solutions were mixed in the given volume mixing ratios. A 1 μ L aliquot of the solution was hand-spotted on a stainless steel target and allowed to dry by air. Hardly soluble components in a specified solvent were

Table 1. Volume mixing ratios of the 2³ full factorial design with triplicated center point for three experimental factors: volume of polymer solution (P), volume of matrix solution (M), and volume of cationization reagent solution (CR). For the investigated polystyrenes and PDMS1110, the experimental design was applied to all 240 combinations (eight matrices, five salts, and six solvents) whereas for the other examined polymers 40 to 80 combinations were studied

Run	V(P)	V(M)	V(CR)
1	2.5	28	5
2	5	5	10
3	1	5	10
4	1	5	1
5	1	50	1
6	2.5	28	5
7	5	50	10
8	1	50	10
9	5	5	1
10	5	50	1
11	2.5	28	5

made as saturated solutions at ambient temperature and did not correspond to the above mentioned concentrations.

Experimental Designs

First the suitability of the three different experimental designs was investigated in over 100 experiments. Four measurements of each sample were carried out for evaluating standard deviation. For multiple response optimizations, molecular weight at maximum of distribution (M_p), signal intensity, signal-to-noise (S/N) ratio, and spectroscopic resolution were chosen as response variables; the latter three variables were taken from M_p . Additionally, two response variables were worked out describing the quality of the distribution: the number and intensity of interfering peaks (caused by clusters or further peak series besides the principal series) were taken between M_p and $M_p + 1$ repeat unit. The number of interfering peaks is gathered simply: one counts the interfering peaks whose signal intensity is more than 2% of the M_p peak, so that values between 0 and 4 are obtained (if there are more than four peaks the value is still 4). For evaluating the intensity of interfering peaks only the one with the highest signal intensity was considered. The values for the intensities are classified from 0 to 5: 0 when no interfering peak is observed; if the intensity of the interfering peak was less than 10% of the M_p , the intensity was evaluated by 1; 2 represents interfering peak intensities of 10 to 40% of M_p ; 3 represents interfering peak intensities of 40 to 70% of M_p ; 4 represents interfering peak intensities of 70% to 100% of M_p ; a value of 5 represents samples whose interfering peaks were higher than those of the examined distribution. It must be emphasized that particularly for lithium trifluoroacetate LiTFA, an additional adduct peak series of [polymer + Li(LiTFA)]⁺ was observed aside from the principle peak series [polymer + Li]⁺. Depending on the employed mixing ratio and

combination the adduct peak series possessed higher signal intensities than the principle peak series. Such TFA-adduct peak series were also obtained for copper trifluoroacetate as well but they did occur more rarely. For silver trifluoroacetate a broad foot of the peak to the right side or a split peak is observed showing that the adduct peak series is ubiquitous. The higher the values of the number and the intensity of interfering peaks the worse is the mass spectrum.

The chosen response variables show variations in molar mass distributions caused by different mixing ratios. All experimental designs were calculated and evaluated using STATGRAPHICS Centurion XV (StatPoint, Inc., Warrenton, VA, USA). Furthermore, a multifactor analysis of variance (ANOVA) was carried out investigating trends and highlighting particular results.

Results and Discussion

Design of Experiments

Three different experimental designs were investigated for their feasibility on studying the effect of mixing ratio on molar mass distributions of synthetic polymers. Usually, experimental factors are investigated in screening designs to find the significant ones. In this work only three experimental factors—volume of polymer solution, volume of matrix solution, and volume of cationization reagent solution—were investigated so that the selected experimental designs could be run in a random way.

The Box-Behnken design is a response surface design where the three experimental factors can be studied in one block of 15 runs including three center points. The design has been fully randomized. Besides this response surface design, two screening designs were investigated. The 2^3 full factorial design was used to study the effects of the three factors in one block of 11 runs with triplicated center points spaced in the randomized design. To reduce the number of runs a 2^{3-1} fractional factorial design of six runs including two center points was created.

The Box-Behnken design gave comparable results as the 2^3 full factorial design, but contains a higher number of runs. By contrast the number of runs of the 2^{3-1} fractional factorial design is not sufficient: for some combinations only at one or two mixing ratios of the full factorial design reliable MALDI mass spectra were obtained. If these mixing ratios were not considered in the fractional factorial design no reliable MALDI mass spectrum would be obtained and the investigated combination would be stated to have failed. With the employed fractional factorial design most samples were correctly optimized but failed in a few cases. According to these findings the 2^{3-1} fractional factorial design is not suitable for studying the effect of mixing ratio. Both Box-Behnken design and full factorial design are suitable for studying the effect of molar mixing ratios on molar mass distributions but the full factorial design is

more economic due to its lower number of runs. In the following sections only results obtained with the full factorial design are discussed.

The Effect of Mixing Ratio

A reliable mass spectrum is characterized by the correct repeat unit, a high signal-to-noise ratio, a high spectroscopic resolution, preferably no interfering peaks and an acceptable repeatability. All of these features are influenced by the mixing ratio. Employing the single solvent approach experiments have failed if polymer non-solvents were used. For the solubility of the used matrices and cationization reagents, respectively, different results were obtained depending on the studied combination. For example, chloroform and dichloromethane are *macroscopic* non-solvents for AgTFA, but for polystyrenes excellent mass spectra were obtained for AgTFA-combinations with non-polar matrices (DCTB, DT, DPBD) using dichloromethane while employing chloroform some interfering peaks could appear. Polar matrices as DHBQ and NA frequently show sodium adduct peak series besides the main silver peak series. The amounts of Ag in the AgTFA-saturated solutions in chloroform as well as in dichloromethane were determined by ICP-OES: 0.014 mg/mL Ag were measured in chloroform and 0.74 mg/mL in dichloromethane (the aimed concentration of the AgTFA solution was 0.1 mol/L = 22 mg/mL). Nevertheless, these concentrations are sufficient for ionization.

Investigating the effect of mixing ratio, some observations were made:

- The first step in optimization is the evaluation of the significance of the investigated factors. In this study, the volumes of polymer solution, matrix solution, and cationization reagent solution were examined as factors as well as their two-factor and three-factor interaction effects. For several combinations studied for a defined type of polymer none of the factors were significant while for other combinations the number and the manner of significant factors varied, i.e., whether one of the examined factors is significant depends on the studied combination as well as on the investigated polymer. For example, for PS2850 all combinations including DCTB as matrix and AgTFA as cationization reagent showed a significant influence of the volume of the cationization reagent solution. For this reason, the effect of mixing ratio should be discussed for all three components together: the mixing ratio of polymer/matrix/cationization reagent.
- The most suitable volume mixing ratios for the studied polymers and combinations for the examined concentrations were 5/5/1 and 5/5/10 (P/M/CR, Figure 1). These two mixing ratios represent the optimal mixing ratio of about 70% of the investigated samples. If the matrix and/or the cationization reagent are not completely soluble in the chosen sol-

Figure 1. MALDI-TOF mass spectra of PS2850 and PS11600 with the combination DT/AgTFA/THF using different volume mixing ratios (P/M/CR) showing the influence of mixing ratios on the same polymer as well as the effect of the molar mass of the polymer.

vent, a higher amount is necessary for obtaining a reliable mass spectrum. For Ag-combinations dissolved in dichloromethane 5/5/1 was the most frequent optimal mixing ratio. By contrast, 5/5/10 was the most frequent optimal mixing ratio using chloroform as solvent. These results were confirmed by the determined Ag-amounts by ICP-OES. Comparing DT and POPOP using THF as solvent different optimal mixing ratios were obtained for polystyrenes due to the lower solubility of POPOP in THF. For DT-samples 5/5/1 is the most frequent optimal volume mixing ratio whereas of POPOP a higher amount is required in the mixture. The optimal volume mixing ratios for POPOP containing samples using THF were 1/50/1 and 5/50/1 (P/M/CR).

- According to the previous paragraph, comparisons of the suitability of different matrices, cationization reagents, and/or solvents, have to be based upon the optimal mixing ratio.
- In Figure 1 the MALDI mass spectra of PS2850 and PS11600 are shown for the combination DT/AgTFA/THF, a frequently used combination for polystyrenes. The shape, the width, the location along the m/z axis (M_p), and the intensity of the polymer distribution is affected by mixing ratio. The extent depends on the polymer, its molar mass, and the studied combination.
- Varying M_n and M_w values were obtained due to varying M_p values, signal intensities, and width of the distribution. For example, for PDMS1110 similar signal intensities were obtained for the volume mixing ratios 5/5/10 and 5/5/1 (P/M/CR) using the combination NA/AgTFA/DMC (dimethyl carbonate, DMC). For the mixing ratio 5/5/1 about 20 peaks may be included in the calculation of the average molecular weights M_n and M_w due to the broader distribution while for the mixing ratio 5/5/10 only few peaks could be incorporated. The determined M_n value of 5/5/1 (1379 Da) is about 21.5% higher than the M_n value yielded at the mixing ratio of 5/5/10 (1083 Da); the M_w values differ about 24.6%.

- According to these findings, “correct” values of the molecular average weights do not exist since hard and fast rules of calculation are not given: for the determination of the average molecular weights of a particular polymer it should be defined whether the average molecular weights are calculated as average values over many mixing ratios or if only one mixing ratio for a given combination is included in calculation. Corresponding to the choice of the combination and mixing ratio the average molecular weights can reach a desired value.
- The occurrence of interfering peaks can be controlled by the mixing ratio. Silver clusters as well as additional adduct peak series can be reduced or eliminated by changing the mixing ratio. This effect is demonstrated for PS2850 in Figure 1: for samples containing high volumes of cationization reagent solution and/or matrix solution silver clusters were observed. Only at the volume mixing ratio 5/5/1 (P/M/CR) a good quality MALDI mass spectrum of PS2850 is obtained without silver clusters.
- Different baseline elevations in the low mass tail were described in literature due to different solvent mixtures [9] and due to different matrices as well [30]. The obtained MALDI mass spectra of this study confirm these observations. Figure 1 shows the effect of the mixing ratio and the molar mass of the polymer on the elevation of the baselines in the low mass tail of the distribution: PS2850 provides flat baselines at all mixing ratios while the baselines elevate in the low mass region for the mass spectra of PS11600 at the volume mixing ratios 5/5/10, 1/5/10, and 1/50/10 (P/M/CR).
- The appearance of multimers or multiply charged molecular ions can also be controlled by the mixing ratio. Our results confirmed the ones of Schriemer and Li [17] who reported that an increased matrix-to-polymer ratio might improve the mass spectra.
- In a few cases the normal polymer repeat unit was determined to be incorrect for most of the mixing

ratios of the full factorial design, but good spectra (with the correct repeat unit) were obtained at one or two mixing ratios.

- The mixing ratio affects the S/N ratios which can be used for quantitation [31]. For explanations see section ANOVA.
- The applied dried-droplet preparation technique often yielded inhomogeneous spots. Depending on the mixing ratio and the combination studied this effect can be reduced.
- Another effect on the spot homogeneity is the precipitation of some matrices with silver or copper ions. Whether this precipitation leads to better or worse mass spectra depends on the studied combination. However, the amount of precipitation can be controlled by the mixing ratio.
- It is well known that laser energy has to be changed with mixing ratio. A higher amount of polymer in the sample requires higher laser energy. The extent of laser energy variation along with mixing ratio depends on the combination studied. For example, the observed variability of laser energy by changing the mixing ratio was higher for combinations with DCTB as matrix than for those with DT.

The applied 2^3 full factorial design was suitable in the entire investigated mass range. Lower molar mass polymers gave good mass spectra at almost all mixing ratios. The impact of mixing ratio increases with increasing molar mass of the polymer, i.e., only one or two mixing ratios of the applied 2^3 full factorial experimental design were suitable for higher molar mass polystyrenes. Many authors stated that a larger amount of matrix should be added to the sample when the molar mass of the polymer increases. The applied experimental design supports this demand since all parameters besides the molar mass of the polymer remain constant: using the same volume mixing ratios the molar matrix/polymer ratio increases with increasing molar mass of the polymer.

Analysis of Variance (ANOVA)

For each polymer, a multifactor ANOVA was carried out investigating the effect of mixing ratio on different response variables. Furthermore, several multivariate ANOVA statistics were calculated for this effect. As response variables for the ANOVA M_p value, S/N ratio, signal intensity, laser energy, spectroscopic resolution, and both variables describing the amount of interfering peaks were chosen. All these variables represent the quality of the spectra and therefore the suitability of the studied combination. The main effects of mixing ratio, matrix type, cationization reagent, and solvent as well as the two-factor interaction effects and three-factor interaction effect of matrix type, cationization reagent, and solvent, were modeled in the ANOVA.

The ANOVA results were presented for PS2850 and PDMS1110 for M_p values and signal-to-noise ratios. For

PS2850 240 samples were analyzed with the 2^3 full factorial design. These 240 samples were made up of all combinations of eight matrices (DCTB, DHAP, DHB, DHBQ, DPBD, DT, NA, and THAP), five cationization reagents (AgTFA, LiTFA, $\text{Cu}^{(II)}$ TFA, NaNO_3 , and LiCl), and six solvents (THF, toluene, chloroform, dichloromethane, methyl ethyl ketone, and methyl isobutyl ketone). For the multifactor ANOVA 72 of the 240 combinations were evaluated. This data diminishment was done to reduce the solubility impact on the ANOVA statistics. Because of the poor solubility or non-solubility of NaNO_3 and LiCl in the chosen solvents poor mass spectra were obtained or the analysis had failed. Likewise observations were made for combinations of LiTFA and $\text{Cu}^{(II)}$ TFA in chloroform, dichloromethane, and toluene. Since these combinations may have a significant influence on the ANOVA they were not considered. The 72 samples of PS2850 for the ANOVA were made up of all combinations of AgTFA, $\text{Cu}^{(II)}$ TFA, and LiTFA as cationization reagents, the eight matrices, and THF, methyl ethyl ketone, and methyl isobutyl ketone as solvents ($3 \times 8 \times 3 = 72$). A likewise data reduction was done for PDMS1110. Although 240 combinations were tested only 64 combinations could be included in the ANOVA. The 64 samples of PDMS1110 were made up of all combinations of the 8 matrices, AgTFA and LiTFA as cationization reagents as well as THF, methyl ethyl ketone, dimethyl carbonate, and 1,4-dioxane as solvents ($8 \times 2 \times 4 = 64$).

For both examined polymers the most significant factor, according to Wilks' lambda of the MANOVA statistics, is the three-factor interaction effect of matrix, cationization reagent, and solvent. Hence, the entire combination employed for one polymer has a higher impact on the molar mass distribution than the mixing ratio. However, the P values of the effect of mixing ratio on all considered response variables are less than the significance level of the risk taken to be 0.05, i.e., there is a statistically significant effect at the 95% confidence level. Great variations are observed for S/N ratios along with the mixing ratio in which the mixing ratios with the higher volume of the polymer yielded higher S/N values for both examined polymers. For PS2850 the S/N ratio decreases with increasing volumes of matrix solution as well as cationization reagent solution at constant volume of polymer solution (Figure 1) while for PDMS1110 at the higher volume of cationization reagent solution the higher S/N ratios were obtained at constant polymer/matrix ratio. High S/N ratios were yielded at the volume mixing ratios 5/5/1 and 5/5/10 (P/M/CR) which were found to be the best for the most investigated samples. The M_p values vary within two repeat units for both polymers. The highest M_p values were obtained at a high amount of matrix in the mixture; 1/50/1 for PS2850 and 5/50/1 for PDMS1110.

The ANOVA and MANOVA statistics confirm the above mentioned observations: the mixing ratio affects the M_p values and, hence, M_n as well as M_w values, S/N ratios, signal intensity, resolution, and the manner of

interfering peaks; laser energy has to be adjusted for each mixing ratio. The optimal mixing ratio itself depends on the entire combination studied. These results are the same for the other investigated polymers, i.e., PEG and PMMA.

Conclusions

The investigation shows that the employed 2^3 full factorial design is suitable for the study of the effect of mixing ratio. Furthermore, this experimental design serves as a basis for the variation of the mixing ratio at the given initial concentrations of the polymer solution, the matrix solution, and the cationization reagent solution. Many combinations (matrix, cationization reagent, solvent) were investigated for several synthetic polymers using the full factorial experimental design. The obtained mass spectra as well as the ANOVA results show that the mixing ratio affects the molar mass distribution and its influence depends on the combination studied. For the employed synthetic polymers and combinations the most frequent optimal volume mixing ratios are 5/5/1 and 5/5/10 (P/M/CR) for the studied concentrations. When comparing matrices for the determination of synthetic polymers, it is mandatory to take the cationization reagent, solvent, and mixing ratio into account. Only optimized conditions should be compared; otherwise there will be the risk of erroneous interpretations.

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